Peer Review File

Article information: http://dx.doi.org/10.21037/pcm-20-62

Response to Reviewers' Comments:
We thank the Reviewers and Editor for their feedback and helpful comments. We have addressed specific points as follows and have included revisions in the manuscript document (highlighted).

Reviewer A

Comment 1: "*It is unclear why the authors state that the effect of erlotinib on EGFR phosphorylation was unexpected since this was observed by Guerrab et al in Oncotarget in 2016. The finding that there was a difference in EGFR expression in 468 vs 231 cells was also shown by this group. The effect of erlotinib on cell viability has similarly been shown in TNBC cells, however, this group showed that both 231 and 468 were responsive to erlotinib. The authors need to address what is new/different in their analysis and provide some potential explanations. There is not the same concern for the c-MET part of the paper".*

Reply 1: We thank the reviewer for this comment and agree that our findings with regards to the observed endogenous levels of EGFR in the two cell models and the observation that erlotinib reduces EGFR phosphorylation support the findings of previous studies. However, it was the effect of erlotinib on receptor expression (not just phosphorylation) of EGFR in both MDA-MB-231 and MDA-MB-468 cell models that was unexpected and novel, as this is not the canonical targeting mechanism of erlotinib and has not previously been reported in triple-negative breast cancer. This point has been further highlighted in the Discussion (Lines 308-316, 347-333 in the revised manuscript.

Comment 2: "*Figures in general- please present the 468 and 231 data in the same order in all panels to make things easier for the reader (eg. figure 1 and 3; panel C vs panels D and E".*

Reply 2: We thank the reviewer for catching this error. We have now corrected this in Figure 1 and 3 in the revised manuscript such that the MDA-MB-468 and MDA-MB-231 data is presented in the same order in all panels.

Comment 3: "*Figure 4 - Some of the blots are too dark to be able to see any difference (ie. Panel A- phospho-MET, Panel D- phospho-ERK). In the case of panel D, the small decrease in P-Akt (maybe?) in combination with the increased level of total Akt should have resulted in a decrease for both phosphorylation sites but without a lighter exposure with more contrast it is impossible to tell".*

Reply 3: The quantified phosphorylation changes reported in Figure 4A, 4B, and 4D are the result of compiled densitometric immunoblot analysis (n=3 for each experiment). In the revised manuscript we have presented representatives blots with a more optimized exposure.

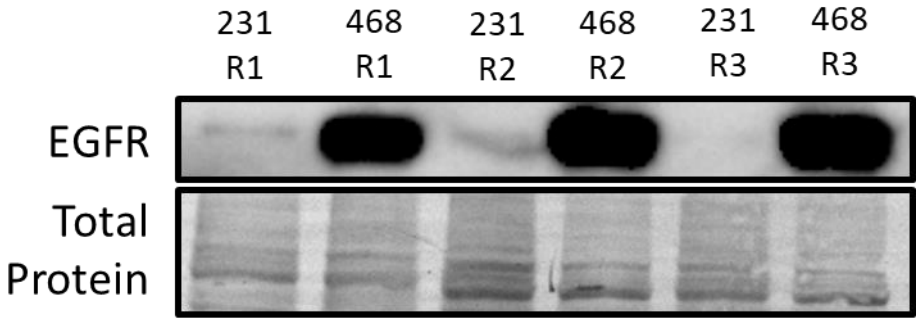
Comment 4: "*Typo on page 19- phosphor instead of phosphor"*

Reply 4: We thank the reviewer for catching this, and we have now corrected this in the revised manuscript (Line 350).

Reviewer B

Comment 1: *"WB of EGFR in figure C showing that MDA-MB231 is negative for EGFR is just unrealistic".*

Reply 1: In Figure 1C, the expression of EGFR in MDA-MB-231 cells was quantified on the same immunoblots (n=3) in comparison to the expression in MDA-MB-468 cells, both in the presence of EGF stimulation, with a representative blot show. It has long been known in the literature that the MDA-MB-468 cell line overexpresses very high levels of EGFR (Filmus et al., 1985*). Since the expression of EGFR in MDA-MB-468 cells is over-expressed a high levels in comparison to MDA-MB-231 cells, a high level of overexposure is necessary to detect low levels of EGFR in the MDA-MB-231 cells. We re-ran the 3 experimental sample sets together and show the overexposed immunoblot image to demonstrate this.



*Reference: Filmus J, Pollak MN, Cailleau R, Buick RN. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Commun. 1985 Apr 30;128(2):898-905. doi: 10.1016/0006-291x(85)90131-7. PMID: 2986629.

Comment 2: "*For all WB used, loading control should be used such as Actin or GAPDH, HSP70 or HSC70".*

Reply 2: Although actin and other housekeeping proteins are commonly used as loading controls for immunoblots when only one cell line is being analyzed, we respectfully feel that total protein staining (e.g. Amido Black as used/shown) is a more appropriate loading control for our experiments. This is a well-accepted method of loading control, particularly when different cell lines are analyzed together and/or treatment conditions involve drugs that may affect housekeeping pathways as well as target pathways. In our experience, the expression of housekeeping proteins can be variable between cell lines and the expression may be subject to change between treatment

conditions. Total protein staining, as loading control, provides a more accurate measure of total protein loaded per lane and is more reliable measure to use for normalizing protein changes when quantifying immunoblots. (Aldridge et al, 2008*). In summary, the unreliability/uncertainty in expression of housekeeping proteins do not make them appropriate loading controls for our experiments, and thus we used Amido Black total protein staining.

*Reference: Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. J Neurosci Methods. 2008 Jul 30;172(2):250-4. doi: 10.1016/j.jneumeth.2008.05.003. Epub 2008 May 15. PMID: 18571732; PMCID: PMC2567873.

Comment 3: "*It is not clear why EGFR is downregulated by Erl in Figure 2A"*

Reply 3: We agree with the reviewer that the downregulation of EGFR expression in response to erlotinib treatment was unexpected. Although we carried out initial rescue experiments for EGFR expression by inhibiting the lysosomal and proteasomal pathways the two most cited pathways of stress-induced EGFR downregulation (Figure S1C-E), this was not the mechanism of action in our cell models. Future investigation of this will be important, although it is beyond the scope of the current manuscript. In the revised manuscript, we have now included discussion of some potential mechanisms which may be contributing to this novel observation (Lines 308-316; 327-333).

Comment 4: "*The fact that cabozantinib has no effect on c-MET means that this pathway is not involved in MDA-MB231 cell phenotype*"

Reply 4: We agree with the reviewer that cabozantinib may be affecting the MDA-MB-231 cells through a pathway regulated by a target that isn't c-Met. However, cabozantinib still presents an important inhibitor to study in triple negative breast cancer as it attenuates proliferation, migration, and invasion of responsive breast cancer cells, likely through its inhibitory effects on the AKT pathway. We have added to the Discussion (Lines 350-355) in the revised manuscript to highlight this point.